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Identification of genes differentially expressed in mouse mammary epithelium transformed by an activated β-catenin

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β-Catenin is an executor of Wnt signaling and it can control cell fate and specification. Deletion of exon 3 from the endogenous *β-catenin* gene in differentiating mammary alveolar epithelium of the mouse results in the generation of an activated protein that lacks amino acids 5–80. This is accompanied by a loss of mammary epithelial differentiation and a transdifferentiation process to squamous metaplasias. To further understand the molecular process of transdifferentiation, the expression of genes in mammary tissue was profiled in the absence and presence of activated of β-catenin. Microarrays were generated that carry about 8500 cDNA clones with approximately 6000 obtained from mammary tissue. Mutant tissues, which had undergone either partial (TD1) or complete (TD2) squamous transdifferentiation, were compared with wild-type mammary tissue. Four groups of genes were identified. Group 1 contained genes whose expression was induced in both mutant tissues. Groups 2 and 3 contained genes that were active preferentially in TD2 and TD1, respectively. Group 4 contained genes suppressed in both samples. Using this approach, known and unknown genes activated in the transdifferentiation process were identified. A new 20 kDa protein (PANE1) induced upon transdifferentiation was nuclear in nonconfluent cells and cytoplasmic in confluent or dividing cells. Lastly, stabilization of β-catenin resulted in the retention of differentiated epithelium upon involution and altered activities of several proteases in transdifferentiated mammary epithelium.
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Introduction

β-Catenin is a multifunctional protein capable of connecting cadherins to the actin cytoskeleton via α-catenin (Bullions and Levine, 1998), and it has a critical role in the Wnt signaling pathway. In the absence of Wnt signaling, β-catenin is bound by the adenomatous polyposis coli (APC) tumor suppressor and either AXIN1 or AXIN2 together with glycogen synthase kinase 3β (GSK3β), which phosphorylates specific serine and threonine residues in the N-terminal domain. Phosphorylated β-catenin is destined for proteasome-mediated degradation. Following association of extracellular Wnt with its transmembrane receptor Frizzled and coreceptor LRP, GSK3β is inactivated, which leads to the accumulation of β-catenin and its translocation to the nucleus (Polakis, 2000). There β-catenin associates with transcription factors of the TCF family (Brantjes *et al*., 2002) and activates target genes involved in transcription, cell cycle regulation, apoptosis and matrix remodeling. Mutations and deletions of phosphorylation sites in the amino terminus result in the stabilization of β-catenin and have been identified in human tumors and cell lines (Polakis, 2000). Elevated levels of β-catenin have been associated with poor prognosis in human adenocarcinomas of the breast (Lin *et al*., 2000). Expression of an N-terminally truncated β-catenin under the control of mouse mammary tumor virus-long terminal repeat (MMTV-LTR) sequences resulted in aberrant alveolar budding, precocious alveolar development and in the formation of mammary adenocarcinomas (Imbert *et al*., 2001; Michaelson and Leder, 2001).

Recently, the stabilization of β-catenin through Cre-mediated deletion of exon 3 (amino acids 5–80) in differentiating mammary alveolar epithelium has been demonstrated to induce transdifferentiation into epidermal-like structures, squamous metaplasias and pillar structures (Miyoshi *et al*., 2002). Loss of exon 3 of the *β-catenin* gene in differentiating mammary epithelium was achieved with a Cre transgene under control of the *WAP* gene promoter. Accumulation of β-catenin led to a loss of epithelial differentiation followed by a prominent transdifferentiation into squamous metaplasias. On a histological level, the accumulation of β-catenin led to

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the loss of Stat5a, NKCC1 and Npt2b, with increased levels of cytokeratins K1 and K6 heralding the squamous transdifferentiation.

There is a paucity of molecular insight into the process of transdifferentiation, and this study focused on the elucidation of its molecular consequences. Towards this end, gene expression profiles for normal mammary epithelium and β-catenin-induced squamous metaplasias were established. To accomplish this goal cDNA microarrays, including one specifically designed for mammary tissue (Mammochip), were generated. The gene expression profiles were investigated in samples displaying different stages of metaplastic progression versus normal mammary tissues. New and known genes showing differential expression patterns between normal mammary tissue, squamous metaplasia and skin have been identified.

Results

Stabilized β*-catenin induces transdifferentiation of mammary epithelium*

Previously, our lab reported that the stabilization of endogenous β-catenin in differentiating mouse mammary epithelium leads to its transdifferentiation into epidermal-like structures (Miyoshi *et al*., 2002). In order to better understand the genetic steps that lead to the loss of mammary epithelial differentiation, the acquisition of epidermal features and the transdifferentiation into squamous metaplasias, we attempted to identify those genes that were repressed or activated upon the stabilization of β-catenin. For this purpose, Catnb$^{+/\Delta ex3}$:WAP-Cre mice were bred and two stages of transdifferentiation were chosen (Figure 1). The first sample (TD1) was harvested after the first pregnancy at 10 weeks of involution. It featured keratinized squamous metaplasias as well as structures resembling condensed alveoli and stromal components. At a more advanced stage (TD2) from a different animal at the same time point, very few alveolar structures had remained and the majority of the epithelium had undergone squamous transdifferentiation. The histology from each tissue was used to assign the relative stage of transdifferentiation (TD1 had less transdifferentiation than TD2). In contrast, wild-type mammary tissue from mature virgins and during involution exhibited a normal ductal structure embedded in the stroma.

Gene expression in mammary tissue from wild-type virgin, lactating and involuted mice

The mouse Mammochip was used to examine the profile of mRNA expression in virgin, lactating and involuted tissues. This chip was developed by our laboratory and contains approximately 6000 cDNA sequences isolated from different mammary-specific libraries (see Materials and methods). To validate the Mammochip, gene expression profiles from virgin, lactating and involuted mammary tissue were compared. Complementary DNA prepared from age-matched wild-type virgin mammary tissue (wtV) was used as a common partner and hybridized with cDNA prepared using mammary tissue from wild-type lactating (wtL) and involuted (wtI) mice. Out of more than 6000 cDNAs, 315 (78 known genes) were differentially expressed over threefold between the virgin and lactating states (Table 1). As expected, the

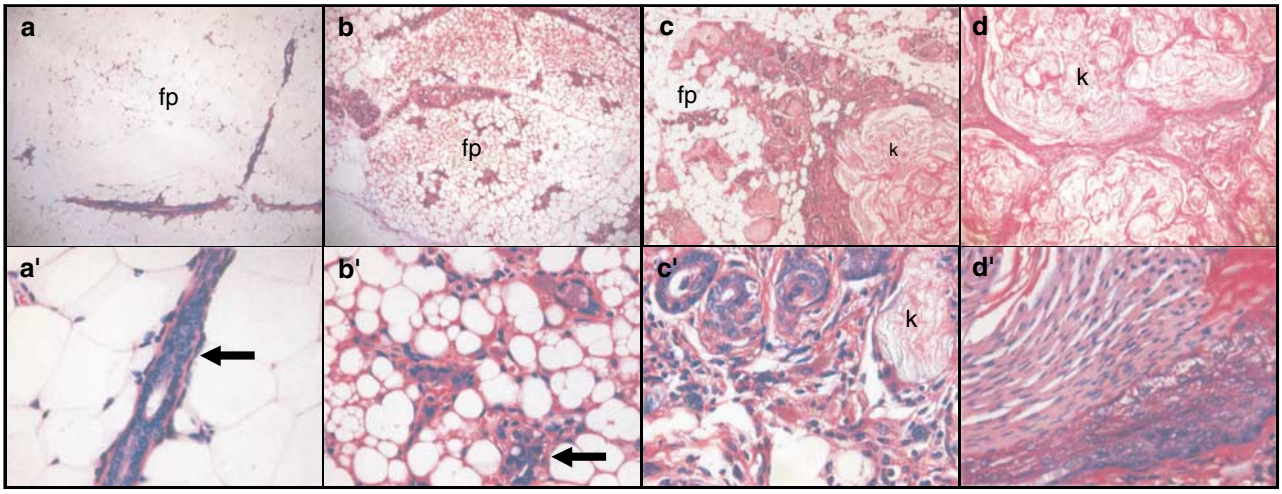

Figure 1 Histological sections of mammary tissues used in microarray analyses. (**a**) Mammary tissue from 10-week-old virgin exhibiting a fat pad with some ductal structures. (**a′**) Higher magnification of (**a**) showing ductal structure (arrow). (**b**) Mammary tissue from a wild-type mouse at 10 weeks of involution, which has undergone extensive remodeling and has the appearance of mature virgin tissue. (**b′**) Higher magnification (**b**) with an area including a remodeled alveolar structure (arrow). (**c**) Mammary tissue from a Catnb$^{+/\Delta ex3}$:WAP-Cre mouse that had gone through one pregnancy and 10 days of involution (sample TD1). Epithelial structures, a stromal compartment and squamous metaplasias are visible. (**c′**) Higher magnification of (**c**). (**d**) Mammary tissue from a Catnb$^{+/\Delta ex3}$:WAP-Cre mouse from the same time point as (**c**) that displayed a higher degree of transdifferentiation (sample TD2). Epithelial structures, a stromal compartment and squamous metaplasias are visible. Most of the tissue had undergone sqamous transdifferentiation and no significant amounts of epithelium had remained. (**d′**) Higher magnification of (**d**). (fp) indicates the mammary fat pad and (k) indicates keratin deposition

Table 1 Genes differentially expressed over 3 fold between lactating and virgin wild-type mammary gland

		L/V
IMAGE:851207	arsenate resistance protein 2	90.02
IMAGE:875031	casein kappa	82.05
IMAGE:874488	casein alpha	97.48
IMAGE:1314775	casein beta	95.79
IMAGE:1054751	casein gamma	89.83
IMAGE:1511486	whey acidic protein	87.59
IMAGE:1511509	PDZ protein interacting specifically with TC10	38.83
IMAGE:1400768	RAB18, member RAS oncogene family	36.11
IMAGE:875062	apoptosis-associated tyrosine kinase (AATYK)	34.97
IMAGE:1511382	casein delta	29.45
IMAGE:832158	extracellular proteinase inhibitor (WDNM1)	14.92
IMAGE:1400441	E74-like factor 5	13.81
IMAGE:1510567	serum amyloid A 3	10.89
IMAGE:891142	lysosomal acid lipase 1	9.92
IMAGE:832408	AXL receptor tyrosine kinase	5.65
IMAGE:831635	cholecystokinin	5.40
ESTs	IMAGE:1430589, 851337, 1247661, 820682, 1494877, 1511559, 832278, 1245686, 1346211, 1054643, 1448138, 1247736, 1397838, 1246820, 1448074, 874482, 1248573, 1314860, 891214, 1067847, 1054324, 1430609, 1381948, 949540, 1448100, 1054043, 1494871, 1511173, 1395749, 1510610, 1510950, 1511503, 1495042, 1245832, 1067103, 851209, 1384186, 1245672, 963526, 1348833, 1245783, 1382204, 1364090	
IMAGE:875481	UDP-Gal:betaGlcNAcb 1,4- galactosyltransferase	4.92
IMAGE:1245854	endomucin	4.36
IMAGE:1313559	mucin 1, transmembrane	4.12
IMAGE:1382671	programmed cell death 4 (MA-3)	4.10
IMAGE:832113	adipose differentiation related protein	3.68
IMAGE:1245706	sim to sialyltransferase 1	3.62
IMAGE:874596	meningioma expressed antigen 5 (hyaluronidase)	3.42
IMAGE:1247703	angiotensin converting enzyme	3.34
IMAGE:1195103	FXYD domain-containing ion transport reg. 3	3.20
ESTs	IMAGE:949074, 1510672, 1384121, 1448316, 949854, 873929, 864395, 1248009, 1448943, 1384047, 1477122, 1476966, 891107, 863639, 1247699, 1398298, 1195127, 1495974	
IMAGE:1230503	phosphatidylcholine transfer protein	0.33
IMAGE:890670	phosphodiesterase 1B, Ca2+-calmodulin dep.	0.33
IMAGE:1398011	guanine nucleotide binding protein (G protein), γ10	0.32
IMAGE:891323	hypothetical protein MGC7673	0.32
IMAGE:876909	solute carrier family 28 (Slc28a2)	0.32
IMAGE:1245498	Tnfa-induced adipose-related protein	0.31
IMAGE:820132	LIM only 2	0.31
IMAGE:1179007	hypothetical protein, MNCb-5210	0.31
IMAGE:1382855	amyloid beta (A4) precursor-like protein 2	0.31
IMAGE:1068616	oxysterol binding protein homolog 1	0.31
IMAGE:1054843	reversion-inducing-cys-rich prot. with kazal motifs	0.30
IMAGE:832253	slit homolog 3 (Drosophila)	0.30
IMAGE:1383614	insulin-like 6	0.30
IMAGE:949230	N-myc downstream regulated 2	0.30
IMAGE:948835	cyclin-dependent kinase 7	0.30

Ratios pseudo-color scale

.2 .33 .5 2 3 5

		L/V
IMAGE:820373	sim to adaptor-related prot. complex AP-3	0.30
IMAGE:1245142	procollagen, type IV, alpha 6	0.30
IMAGE:1245154	interleukin 3 receptor, alpha chain	0.30
IMAGE:876728	claudin 4	0.29
IMAGE:1313957	small inducible cytokine subfamily A, member 22	0.29
IMAGE:935316	E26 avian leukemia oncogene 2, 3' domain	0.29
IMAGE:1054503	nuclear receptor subfamily 1, group I, member 3	0.28
IMAGE:1314017	isocitrate dehydrogenase 3 (NAD+) alpha	0.28
IMAGE:851348	molec. possessing ankyrin-repeats induced by lps	0.27
IMAGE:1247935	origin recognition complex, subunit 4	0.26
IMAGE:1265649	malic enzyme, supernatant	0.26
IMAGE:1068098	zinc finger protein 106 and calpain (Capn3)	0.25
IMAGE:1265616	angio-associated migratory protein (AAMP)	0.25
IMAGE:1314022	lipoprotein lipase	0.24
IMAGE:1177749	pre T-cell antigen receptor alpha	0.24
IMAGE:1247905	small inducible cytokine B subfam., mb 9	0.23
IMAGE:1068786	nucleolar protein GU2	0.22
IMAGE:876418	ATP-binding, sub-fam. D memb.4	0.21
IMAGE:1066861	8-oxoguanine DNA-glycosylase 1	0.21
ESTs	IMAGE:1248105, 1195450, 1067531, 820233, 963535, 1494796, 1246128, 949592, 1400033, 1396558, 1477353, 1247529, 1396449, 1248163, 1430633, 949701, 832131, 875024, 948442, 890568, 935531, 1314940, 1494848, 1068124, 891453, 1349720, 1067780, 1477336, 1066855, 850967, 1244561, 947715, 1314650, 1365243, 876530, 1314067, 863024, 820139, 876902, 875125, 1244964, 1349255, 1178983, 1066847, 876220, 1195620, 1449092, 949593, 1349472, 876625, 948774, 832048, 1244869, 876479, 1495141, 1067317, 863633, 1495233, 1068653, 1495123, 1245495, 820226, 873874, 1195532, 820307, 1195467, 1348287, 863853, 863377, 876068, 1314494, 1494744, 876329, 1245619, 1314677, 891226, 891386, 863308, 853253, 1245272, 850556, 1314249, 1347541, 1400024, 1348682, 875993, 948600, 850165, 1245974, 876374, 948585, 891400, 863283, 874657, 1398261, 1245109, 832458, 1313987, 1068850, 1178993, 890825, 1244922, 863670, 1314112, 1245164, 832481, 1313992, 1314087, 1066706, 850550, 1314031, 1399222, 1314079	
IMAGE:876373	faciogenital dysplasia homolog 2 (human)	0.20
IMAGE:1179554	caveolin 2	0.20
IMAGE:891499	PDZ domain containing, X chromosome	0.19
IMAGE:1248540	solute carrier family 1, member 7 (Slc1a7)	0.17
IMAGE:948509	caveolin, caveolae protein, 22 kDa	0.16
IMAGE:1348345	follistatin-like 3	0.15
IMAGE:832109	resistin	0.14
IMAGE:1247485	myosin, heavy polypeptide 4, skeletal muscle	0.14
IMAGE:850835	small inducible cytokine subfamily B, member 15	0.14
IMAGE:851374	stearoyl-Coenzyme A desaturase 1	0.13
IMAGE:863657	hypothetical protein, MGC:7002	0.13
IMAGE:832222	fatty acid binding protein 4, adipocyte	0.12
IMAGE:831931	carbonic anhydrase 3	0.11
IMAGE:874166	fat specific gene 27	0.11
IMAGE:862840	interferon concensus sequence binding protein	0.10
IMAGE:862835	short stature homeobox 2	0.09
IMAGE:1247588	adipocyte complement related protein of 30 kDa	0.09
ESTs	IMAGE:862807, 863604, 1383598, 851715, 1447704, 851739, 1195555, 1496185, 862999, 1477290, 1068765, 1247486, 890550, 1383148, 1244284, 874999, 1398969, 863781, 1177933, 1247911, 851492, 874045, 1247919, 948437, 963845, 831969, 948556, 1247669, 947677, 1366757, 1067531, 1383163, 1383141, 1496210, 1477325, 1067414, 832237, 948415, 1054191, 1494739, 963432, 1477379, 864839, 876369, 963664, 1248141, 1244320, 1066914, 948547, 1245754, 1066860, 1245777, 863361, 820209, 1396418	

expression of milk protein genes, such as those encoding caseins, WAP and WDNM1, was 60–80-fold elevated during lactation. Additional known and unknown genes were identified whose expression was similarly induced during lactation (Table 1). Some of the encoded proteins may be novel milk components while others may represent cytoplasmic proteins involved in the physiology of mammary epithelium. For example, mRNA levels for arsenate-resistance protein 2 (Ars2), a potential tumor suppressor with unknown function (Wang *et al*., 1996; Rossman *et al*., 1997), were induced 90-fold at lactation. Rab18, a small GTPase that regulates vesicular transport with specificity for polarized epithelial cells (McMurtrie *et al*., 1997) was induced over 35-fold in the lactating gland. It can be hypothesized that Rab18 is involved in the differentiation process of secretory epithelium.

Expression of genes associated with fat metabolism was higher in mammary tissue from virgin mice (Table 1). The levels of RNA encoding a diverse set of proteins, including adipocyte complement related protein of 30 kDa (Acrp30) (Scherer *et al*., 1995), resistin (McTernan *et al*., 2002), fatty acid binding protein 4 (FABP4) (Yang *et al*., 1994), fat-specific gene 27 (Danesch *et al*., 1992), stearoyl-coenzyme A desaturase-1 (STE) (Calabro *et al*., 1982), caveolin (Park *et al*., 2001) and carbonic anhydrase III (CA III) (Lynch *et al*., 1993) were 4–10-fold higher in the virgin. This expression pattern reflects to a large extent the relative distribution of various cell types at different stages of development. While virgin mammary tissue consists predominantly of stromal cells and adipocytes, tissue from lactating mice contains mainly epithelium. Approximately 30 ESTs exhibited a highly elevated level of expression in virgin tissue and some of them possibly encode novel fat-related genes.

To validate the microarray data and identify the nature of the unknown genes, the original IMAGE clones, which had been printed on the slides, were amplified and sequenced. Northern blot analyses were performed with probes for α-casein, CAIII, FABP 4 and STE (Figure 2) and the results obtained agreed with the microarray data and further validated the gene profiling approach.

Since the transdifferentiated tissues were obtained from mice that had gone through pregnancy, lactation and involution, control tissue from the same stage was analysed. A comparison between mammary tissue from mature virgin mice and after 10 weeks of involution displayed 52 differentially expressed genes (Table 2). No significant expression of milk protein genes, with the exception of κ-casein, was observed after complete involution and the profiles of known fat markers were identical to that seen in virgin tissue. However, the expression of several genes associated with lactation was observed after 10 weeks of involution. These include ars2 and 10 ESTs, whose corresponding genes are unknown. Equally interesting, the expression of 37 mostly unknown genes did not reach the level observed before pregnancy. Lastly, the expression levels of two unknown genes (IMAGE clones 864796 and 1396803)

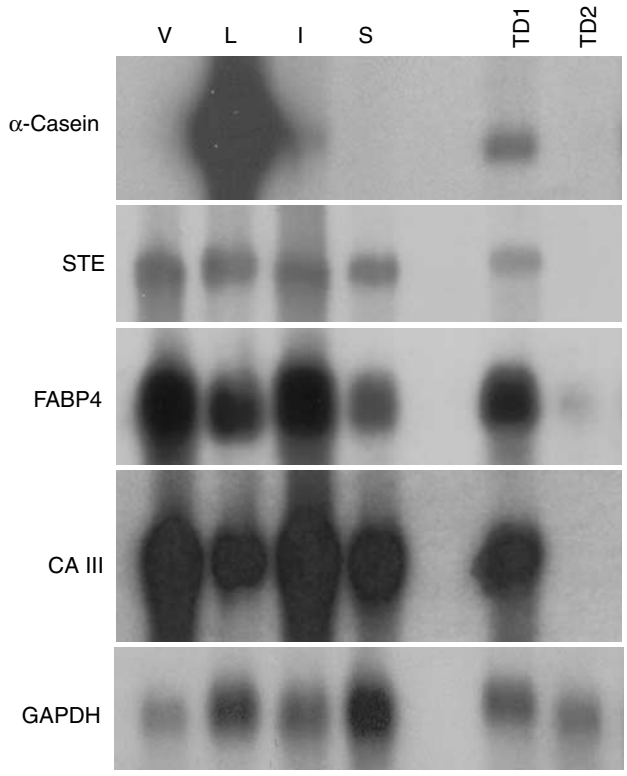

Figure 2 Northern blot analysis with genes whose expression was induced or suppressed in tissue from lactating mice based on microarray assays. Expression of α-casein was detected during lactation and to some extent in tissue sample TD1. Expression of stearoyl coenzyme A desaturase 1 (STE) was lost in the highly transdifferentiated sample TD2. Fatty-acid binding protein 4 (FABP4) and carbonic anhydrase III (CA III) have similar expression patterns as STE. The loss of lipid biosynthesis in the highly transdifferentiated sampe TD2 can be expected because of the replacement of a stroma cell population and apparent decrease in lactation-related milk products. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. V, virgin mammary tissue; L, lactation day 1 mammary tissue; I, day 10 of involution mammary tissue; S, skin; TD1, 'mammary' tissue from $Catnb^{+/\Delta ex3}$:WAP-Cre mice that had gone through one pregnancy and exhibited limited transdifferentiation and remaining epithelial structures; TD2, 'mammary' tissue from $Catnb^{+/\Delta ex3}$:WAP-Cre mice that had gone through one pregnancy and 10 weeks of involution. The TD2 tissue had undergone almost complete squamous transdifferentiation

were higher in the involuted mammary tissue than either virgin or lactating tissue (Table 2, ratios in white). These data strongly suggest that the state of complete involution is distinct from that of a mature virgin, and that the expressed genes reflect the maintenance of remodeled tissue.

Stabilization of β-catenin in mammary epithelium results in the maintenance of expression of lactation-related and the activation of epidermal-specific genes

The gene expression profiles of two distinct stages of mammary tissue that had undergone squamous transdifferentiation were analysed using the NCI Oncochip and the Mammochip. Out of more than 9000 cDNAs, a total of 230 were differentially expressed between wild-

Table 2 Genes differentially over three fold between involuted and virgin wild, type mammary gland, showing their respective expression in the lactating mammary gland

		I / V	L / V
IMAGE:851207	arsenate resistance protein 2	8.08	90.02
IMAGE:1384161	EST	7.86	25.78
IMAGE:1247701	RIKEN gene	6.65	23.35
IMAGE:1495000	EST, common part with casein alpha	6.64	21.39
IMAGE:1245647	RIKEN gene	6.54	48.60
IMAGE:947797	EST	5.50	24.62
IMAGE:1245783	EST	3.29	86.07
IMAGE:875031	casein kappa	4.13	82.05
IMAGE:1364090	EST	4.23	57.61
IMAGE:1495096	EST	4.86	43.53
IMAGE:1382204	EST	3.62	13.72
IMAGE:1348833	EST	3.18	12.31
IMAGE:864796	EST	8.50	
IMAGE:1493858	similar to Hs KIAA1585	4.87	
IMAGE:1396803	WM65 kappa immunoglobulin	4.86	

Ratios pseudo-color scale

.2 .33 .5 2 3 5

		I / V	L / V
IMAGE:874667	EST	0.31	
IMAGE:1245752	EST	0.29	
IMAGE:875174	nuclear cap binding protein subunit 2	0.28	
IMAGE:1349379	EST	0.33	
IMAGE:1069253	EST	0.27	
IMAGE:948781	RIKEN gene	0.28	
IMAGE:1382570	EST	0.31	
IMAGE:1068857	RIKEN gene	0.33	
IMAGE:1430336	RIKEN gene	0.26	
IMAGE:1179057	RIKEN gene	0.33	0.49
IMAGE:1347015	EST	0.28	0.49
IMAGE:1314088	EST	0.29	0.46
IMAGE:851575	EST	0.29	0.46
IMAGE:1068969	EST	0.28	0.46
IMAGE:831836	EST	0.29	0.46
IMAGE:851802	EST	0.31	0.46
IMAGE:875102	RIKEN gene	0.32	0.44
IMAGE:875183	cdc2-inhibitory kinase (Pkmyt1-pending)	0.28	0.43
IMAGE:1178056	RIKEN gene	0.24	0.43
IMAGE:948808	EST	0.30	0.42
IMAGE:875154	Nedd4 WW binding protein 4	0.31	0.41
IMAGE:1054407	EST	0.28	0.41
IMAGE:832244	EST	0.33	0.40
IMAGE:963093	RIKEN gene	0.32	0.40
IMAGE:876798	EST	0.30	0.40
IMAGE:964081	EST	0.33	0.39
IMAGE:1196392	EST	0.29	0.38
IMAGE:875977	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1	0.31	0.37
IMAGE:1314756	calpain 1	0.32	0.37
IMAGE:1245766	parvalbumin	0.31	0.37
IMAGE:1398261	EST	0.25	0.32
IMAGE:832458	RIKEN gene	0.32	0.32
IMAGE:820132	LIM only 2	0.29	0.31
IMAGE:1245109	EST	0.27	0.25
IMAGE:1314022	lipoprotein lipase	0.29	0.24
IMAGE:1179554	caveolin 2	0.31	0.20
IMAGE:1245754	RIKEN gene	0.33	0.14

type and transdifferentiated tissues. While 174 (20 corresponded to known genes) were identified on the Mammochip (Tables 3 and 4), 56 (51 known genes) were identified on the NCI Oncochip (Table 5). The differentially expressed genes were classified in four groups. Group 1 contains genes with an elevated level of expression in both transgenic samples; group 2 contains genes that were activated only in sample TD2; group 3 encompasses genes that were preferentially active in sample TD1; and group 4 contains genes whose expression was reduced in the TD1 and TD2.

Group 1 contained transcripts that were elevated in TD1 and TD2, and it included genes whose expression can be linked to epidermal differentiation and function. Transcripts for keratin 5 and a gene similar to glycolipid transfer protein (GLTP) transcripts were elevated in TD1 and TD2 (Table 3), and also highly expressed in the skin of wild-type mice (Figure 3). Elevated expression of the macrophage expressed gene 1 could be indicative of continued remodeling. Group 3 encompassed genes whose expression was elevated in TD1. These genes include those encoding the milk proteins α, β, and γ-casein (Table 3, group 3). This suggests that the activation of Wnt/β-catenin signaling results in the retention of limited epithelial differentiation even after 10 weeks of involution, a time when normally no significant expression of milk protein genes is observed (Table 2). As expected, expression of these genes was not elevated in sample TD2 as there was no apparent secretory epithelium at this stage (Figure 1).

The expression of milk protein genes would suggest that the Stat5 signaling pathway was still active in the involuted mammary epithelium carrying an activated *β-catenin* gene. Since both Stat5 (Liu *et al*., 1997; Miyoshi *et al*., 2001) and Stat3 (Chapman *et al*., 1999; Humphreys *et al*., 2002) have defined roles in mammary development, we investigated their expression pattern in both the samples. Stabilization of β-catenin led to the loss of Stat5a in the transdifferentiated sample TD2, while it was retained in the less transdifferentiated sample TD1 (Figure 4). Stat3 levels appeared unaltered in TD1 and TD2 compared to mammary tissue and skin (Figure 4). The maintenance of limited Stat5a levels in TD1 suggested the presence of functional alveoli, and the results agree with the expression of milk genes in the less differentiated tissue TD1 and the loss of expression in the highly transdifferentiated tissue TD2. The localization and activity of Stat5a was confirmed by immunohistochemistry (Figure 4). While no nuclear Stat5a was observed in areas of transdifferentiation, nuclear and cytoplasmic staining was observed in the persisting alveolar structures. The presence of nuclear Stat5a may be a consequence of an impaired involution or conversely may be an event subsequent to the activation of β-catenin signaling. The expression of genes indicative of the stromal compartment (fat markers) in virgin and involuted wild-type mammary tissue was lower in both mutant samples (Table 3, group 4), suggesting the decline of a stromal content in the transdifferentiation process.

The gene expression profile of samples TD1 and TD2 was also determined using the NCI Oncochip (Table 5), which contains mostly known genes. Among the activated genes in both transgenic specimen were some encoding proteins involved in tissue remodeling, including prosaposin and cathepsin Z. Northern blot analyses confirmed increased levels of prosaposin and cathepsin Z mRNA in TD1 and TD2 (Figure 3), and zymogram analysis established high levels of cathepsin Z activity in sample TD1 (Figure 6).

Identification of two new genes expressed upon β-catenin activation

The design of the Mammochip included a large number of ESTs without known function, thus favoring the discovery of yet unknown genes. The initial screen included analyses of candidate clones on commercial RNA blots (data not shown) and on RNAs obtained from transdifferentiated tissues (Figure 5). This broad sweep identified two RNA species with interesting expression patterns. IMAGE clone 947797 did not correspond to any known gene, but matched five EST clones from retina, heart and mammary tissue. The size of the RNA transcript was approximately 1.8 kbp and it was detected in lactating mammary tissue and in sample TD1 (Figure 5). Expression was also detected by Northern blot in heart, brain, spleen, liver and kidney (data not shown). IMAGE clone 1348833 had a similar expression pattern to that of caseins, but it did not correspond to any known or predicted gene. It matched to a single genomic locus on mouse chromosome 2 and lined up as four distinct exons. The two unknown genes, although similar in expression to casein genes, are located on separate chromosomes and therefore should not be a secondary effect of activating the casein locus. There was no open reading frame that would produce a protein larger than 39 and 56 amino acids for the IMAGE clones 947797 and 1348833, respectively. These genes may produce large open reading frames after the full-length sequences are determined experimentally, but our results based on a bioinformatic approach did not prompt further analysis for our current study. There is no direct evidence that these expressed RNA sequences display a role in the transdifferentiation process, but they are clearly induced upon the activation of β-catenin.

Identification of a gene encoding a novel 20 kDa protein conserved between species

The expression levels of many unknown genes induced in the transdifferentiated tissues (Table 4) were too low to be detected in the initial screen by Northern blot. However, since some of these proteins may be causally related to the transdifferentiation process, their characterization was pursued. A bioinformatic screen was applied to define a set of genes for further analysis (Table 6, A–C). Each unknown gene from the upregulated list in Table 4 was evaluated for its exon content, open reading frame size and predicted protein

Table 3 Known gnes differentially regulated between transdifferentiated tissues, and wild type involuted mammary gland (Mammochip)

Group 1: Genes highly expressed in both transgenic samples

		TD1/I	TD2/I
IMAGE:820499	RNA polymerase 1-3 (16 kDa subunit)	14.40	16.36
IMAGE:1179525	macrophage expressed gene 1	16.42	9.49
IMAGE:876011	RAB3A, member RAS oncogene family	5.72	8.44
IMAGE:820213	putative GTP binding protein (PGPL)	5.67	5.44
IMAGE:1244500	EH domain-containing protein EHD1	2.34	6.33
IMAGE:863863	keratin 5	3.61	3.90
IMAGE:875002	CD52 antigen	3.19	3.63
IMAGE:1247667	sentrin	2.72	3.44
IMAGE:1448721	aquaporin 5	2.90	3.22
IMAGE:850701	ring-box 1	2.08	3.20
IMAGE:1179487	immunoglobulin heavy chain 6	11.81	2.16
IMAGE:891409	similar to glycolipid transfer protein	3.20	2.08

Group 2: Genes expressed higher in the most transdifferentiated sample

		TD1/I	TD2/I
IMAGE:1510567	serum amyloid A 3		11.72
IMAGE:891142	lysosomal acid lipase 1		6.77
IMAGE:1195105	small inducible cytokine A9		3.50

Group 3: Genes expressed higher in the less transdifferentiated sample

		TD1/I	TD2/I
IMAGE:1314775	casein beta	16.09	
IMAGE:862850	hybridoma 13F1 immunoglobulin heavy chain mRNA	21.46	
IMAGE:874488	casein alpha	10.46	
IMAGE:1493858	similar to Hs KIAA1585	9.55	
IMAGE:1396803	WM65 kappa immunoglobulin	9.11	
IMAGE:1314351	ataxin-1 ubiquitin-like interacting prot.	8.45	
IMAGE:1054751	casein gamma	8.32	
IMAGE:1400768	RAB18, RAS memb.	6.77	

Group 4: Genes with a reduced expression in the transdifferentiated tissues

		TD1/I	TD2/I
IMAGE:850465	calcitonin		0.25
IMAGE:1247485	myosin, heavy polypeptide 4		0.03
IMAGE:949230	N-myc downstream regulated 2	0.33	0.47
IMAGE:948835	cyclin-dependent kinase 7	0.27	0.31
IMAGE:820177	amine oxidase, copper containing 3	0.32	0.30
IMAGE:1247935	origin recognition complex, subunit 4	0.48	0.22
IMAGE:1383614	insulin-like 6	0.46	0.28
IMAGE:876418	ATP-binding cassette, sub-family D (ALD), mb 4	0.42	0.24
IMAGE:851348	molecule posses. ankyrin-repeats induced by lps	0.42	0.26
IMAGE:875108	growth hormone receptor	0.38	0.30
IMAGE:1177749	pre T-cell antigen receptor alpha	0.37	0.21
IMAGE:876313	Sim. to KIAA0761	0.29	0.25
IMAGE:876373	faciogenital dysplasia homolog 2 (human)	0.29	0.21
IMAGE:1068786	nucleolar protein GU2	0.28	0.26
IMAGE:948474	pericentrin	0.26	0.24
IMAGE:1247911	Ewing sarcoma homolog	0.10	0.29
IMAGE:862840	interferon concensus sequence binding protein	0.33	0.20
IMAGE:1066861	8-oxoguanine DNA-glycosylase 1	0.26	0.09
IMAGE:1348345	follistatin-like 3	0.23	0.06
IMAGE:862835	short stature homeobox 2	0.20	0.15
IMAGE:1398011	G protein g10	0.07	0.11
IMAGE:1265649	malic enzyme, supernatant	0.06	0.07
IMAGE:850835	small inducible cytokine subf B, mb15	0.20	0.05
IMAGE:1448821	fatty acid binding protein 4, adipocyte	0.14	0.04
IMAGE:1247588	adipocyte comp. related protein of 30 kDa	0.18	0.04
IMAGE:832109	resistin	0.16	0.03
IMAGE:851374	stearoyl-Coenzyme A desaturase 1	0.20	0.03
IMAGE:1314739	carbonic anhydrase 3	0.17	0.02

Ratios pseudo-color scale

.2 .33 .5 2 3 5

Table 4 Unknown genes differentially regulated between trnasdifferentiated tissues and wild type involved mammry gland (Mammochip)

Group 1: Genes up-regulated in both transgenic samples

		TD1/I	TD2/I
ESTs	IMAGE:87534, 947821, 1511503		
ESTs	IMAGE:820521		
ESTs	IMAGE:1178579, 947854, 1448863		
ESTs	IMAGE:850508		
ESTs	IMAGE:1313772, 947725, 863507, 1496033		
ESTs	IMAGE: 1348833, 1246820		
ESTs	IMAGE:1363898, 1431010, 1496241, 1495233		

Group 2: Genes up-regulated in the most transdifferentiated sample

		TD1/I	TD2/I
ESTs	IMAGE:1399551		
ESTs	IMAGE:1195010, 820678, 875125, 863369, 963317		

Group 3: Genes up-regulated in the less transdifferentiated sample

		TD1/I	TD2/I
ESTs	IMAGE:1447620, 862850, 1384161, 851724, 947797, 1493858		
ESTs	IMAGE:1449282, 1245783		
ESTs	IMAGE:1448100		

Ratios pseudo-color scale

.2 .33 .5 2 3 5

Group 4: Genes reduced in the transdifferentiated tissues

		TD1/I	TD2/I
ESTs	IMAGE:863879, 863516		
ESTs	IMAGE:1245155, 851476		
ESTs	IMAGE:832048, 875024, 1399952		
ESTs	IMAGE:948415, 1494796, 964080, 1494739, 1477379, 1247491, 1496210, 1244320, 1248163, 891453, 963664, 1383148, 1383163, 1247486, 1245754		
ESTs	IMAGE:891096		
ESTs	IMAGE:864839		
ESTs	IMAGE:1348666, 876374, 876375, 1195450, 875993, 948442		
ESTs	IMAGE:1495090, 890550, 891226, 862807, 1195620, 831969		
ESTs	IMAGE:1196365, 863781		
ESTs	IMAGE:1477325, 947715, 1247669, 935531, 851739, 949593, 1448851, 1066914		
ESTs	IMAGE:948509, 1349720, 1247529, 948556, 963535, 1246128, 947659, 863657, 832237, 876369, 851715, 949592, 1067414, 1248075, 1248141, 851492, 963432, 820209, 1396558, 947677, 820233, 1396547, 820307, 1248105, 948437, 863361, 1244284		
ESTs	IMAGE:1195555, 1068765, 863604, 1247919, 948547, 873874, 862999, 1067531, 874999, 874045, 1398969, 1496185, 1195295, 831931, 1383598, 1245777, 1066860, 1477290		

homology. Genes were identified that contained transcripts encoded by several exons, large open reading frames and no homology of the encoded protein to known proteins. Based on the criteria met in Table 6 (A–C) and the conservation between *Homo sapiens*, *Mus musculus* and *Rattus norvegicus* (Table 6D), IMAGE clone 1178579 appeared to be a good candidate for an experimental characterization of the predicted open reading (Figure 6, a–c). For this purpose, a cDNA fragment containing 41–690 bp (GenBank AY243536 and AY243537) from the corresponding RIKEN sequence (clone 2610019I03; GenBank AK011464) was generated and the open reading frame was inserted into a mammalian expression vector. To be able to identify the protein, a C-terminal myc-his tag was inserted. The expression constructs and control vectors were transiently transfected into HeLa cells and incubated for 48 h, followed by protein and RNA analyses and immunofluorescence. Western blot analysis revealed the presence of a protein with an apparent molecular weight of 23 kDa (Figure 6a), which is in agreement with the predicted size of the myc-his fusion protein and the predicted size of approximately 20 kDa for the endogenous protein product. To further verify that the protein expression shown by Western blot was a result of the transfected expression vector, RNA was analysed from native and transfected HeLa cells with a gene-specific probe (Figure 6b). The subcelluar localization of the novel protein was determined by immunohistochemistry using the myc tag for detection (Figure 6c). The protein was located within the nucleus when cultures were not confluent (Figure 6c, a′ and a″), but was excluded from the nucleus upon confluency (Figure 6c, b′ and b″) and in dividing cells (Figure 6c, c′ and c″). The nuclear localization has been confirmed by confocal microscopy (data not shown). There was a predominant exclusion of this protein from the nucleolus when accumulation was detected within the nucleus. There were no known regions of homology with previously described nuclear localization signal peptides as determined using several bioinformatic tools, and at this time no clear transport mechanism may be inferred. Owing to the nuclear localization properties in the subconfluent cultures and the UNIGENE Hs.208912 cluster distribution, we have named this protein PANE1 (proliferation-associated nuclear element). Based on this initial characterization, it is now possible to begin to identify a role for this primary or secondary downstream β-catenin target. The analysis of each new gene expressed in response to the initiation or maintenance of a transdifferentiation process may continue to elucidate signaling events and altered structural components that

Table 5 Genes differentially regulated between transdifferentiated tissues, and wild type virgin mammary glnad (Oncochip)

Group 1: Genes up-regulated in both transgenic samples

		TD1/V	TD2/V
IMAGE:481909	I-kappa B	9.22	15.96
IMAGE:539055	cathepsin Z	2.37	8.07
IMAGE:604444	capping protein beta 1	2.52	7.42
IMAGE:534161	Fc receptor, IgE, high affinity I,	3.66	6.19
IMAGE:406866	prosaposin	3.46	6.14
IMAGE:617054	myosin light chain kinase (Mylk)	2.42	5.66
IMAGE:478473	milk fat globule-EGF factor 8 protein	2.39	4.05
IMAGE:617853	annexin A1	4.43	3.33
IMAGE:425563	transcytosis associated protein p115	3.40	3.23
IMAGE:402270	procollagen, type I, alpha 1	2.55	3.21
IMAGE:480907	transducin-like enhancer of split 1	3.16	3.10
IMAGE:596168	EST	18.41	2.23

Group 2: Genes up-regulated in the most transdifferentiated sample

		TD1/V	TD2/V
IMAGE:553400	Mannosidase 2 B1		29.26
IMAGE:466306	Bone morphogenetic protein 1		8.44
IMAGE:482898	cysteine rich intestinal protein		4.97
IMAGE:536615	melanoma X-actin		4.86
IMAGE:423196	zinc finger protein of the cerebellum 1		3.76
IMAGE:617544	kinase syk		3.51
IMAGE:641615	EST		3.49
IMAGE:559798	PDGF receptor		3.43
IMAGE:373803	actin-related protein 3 homolog (yeast)		3.29
IMAGE:695538	transducer Ras-related		3.03

Group 3: Genes up-regulated in the less transdifferentiated sample

		TD1/V	TD2/V
IMAGE:619073	immunoglobulin joining chain	15.71	
IMAGE:440285	general vav	9.12	
IMAGE:599008	stromal cell derived factor 1	5.56	
IMAGE:617706	EST	4.82	

Group 4: Genes reduced in the transdifferentiated tissues

		TD1/V	TD2/V
IMAGE:481408	cytochrome c oxidase, subunit VIIIa	0.30	
IMAGE:1054099	Trk-fused gene	0.26	
IMAGE:424876	ex matrix fibulin	0.22	
IMAGE:482270	peptidylglycine -amidating monooxygen.	0.21	
IMAGE:568196	hormone/GF IFN a-d Mm.21773 ESTs	0.21	
IMAGE:313981	hemoglobin, beta adult major chain	0.19	
IMAGE:608852	amylase 1, salivary	0.18	
IMAGE:335868	hormone/GF angiotensinogen	0.18	
IMAGE:315777	muscle glycogen phosphorylase	0.16	
IMAGE:573898	Small inducible cytokine A2	0.16	
IMAGE:671218	hormone/GF midkine Mm.906 Midkine	0.15	
IMAGE:337010	makorin, ring finger protein, 1	0.15	
IMAGE:554335	general c-src	0.13	
IMAGE:555498	TF HOX5.4	0.45	0.27
IMAGE:580666	EST	0.44	0.27
IMAGE:574265	RIKEN cDNA 0610009N12 gene	0.36	0.26
IMAGE:660089	effector endozepine	0.35	0.22
MP:8H9	TF BTF3b	0.35	0.25
IMAGE:619026	TF gluc.cor.repr. Mm.23955 ESTs	0.35	0.29
IMAGE:424930	TF erg(ets-like) Mm.29520 ESTs	0.33	0.25
IMAGE:475661	lipoprotein lipase	0.25	0.22
IMAGE:581993	EST	0.38	0.15
IMAGE:316505	myosin light chain, alkali	0.37	0.05
IMAGE:608804	enolase 3, beta muscle	0.33	0.13
IMAGE:608394	hormone melanin conc.	0.33	0.20
IMAGE:659266	general MT2-MMP Mm.25586 ESTs	0.30	0.13
IMAGE:618595	zinc finger protein 26	0.27	0.04
IMAGE:482917	fat specific gene 27	0.22	0.15
IMAGE:478335	retinol binding protein 4, plasma	0.21	0.08
IMAGE:608654	adipsin	0.15	0.10
IMAGE:1314695	serine (or cysteine) proteinase inhibitor	0.15	0.16

Ratios pseudo-color scale

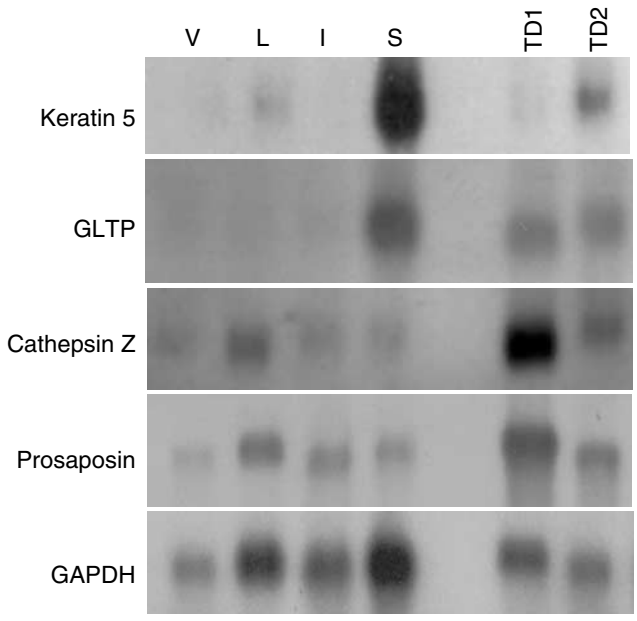

Figure 3 Northern blot analysis of known genes whose expression was altered upon the stabilization of β-catenin based on microarray assays. Keratin 5 expression was higher in sample TD2 than in any stage of mammary development. As expected, its expression was high in skin. Expression of the glycolipid transfer protein (GLTP) was limited to skin and samples TD1 and TD2. Cathepsin Z expression was dramatically increased in the sample TD1 as compared to all of the other tissues analysed, indicating that protease expression may be important during the transdifferentiation process. The pattern of prosaposin expression is similar to that of Cathepsin Z. GAPDH expression was used as a loading control. The tissue samples used were the same as in Figure 2

contribute to the final phenotypic identity expressed by mammary epithelial cells expressing a stabilized β-catenin.

Protease expression in β-catenin induced transdifferentiated mammary epithelium

The microarray analyses revealed the induction of genes related to the establishment and maintenance of the extracellular matrix and tissue remodeling (Table 1). During lactation, the production of procollagen type IV α-6 was reduced (Table 1). Collagen is a common substrate for several proteinases, and the maintenance of the basement membrane in the presence of reduced type IV collagen production indicates a lower level of net protease activity. In transdifferentiated tissues, the level of procollagen type I α-1 mRNA was about threefold higher than in virgin tissue (Table 5). The signals obtained for MT2-MMP and a serine/cysteine proteinase inhibitor were also reduced in the same comparison between transdifferentiated and wild-type virgin samples.

To understand the overall patterns of protease activity, the levels of gelatin and casein zymogen activity were established in transdifferentiated and wild-type tissues using gelatin and casein-embedded Tris-Glycine SDS gels. Increased zymogen activity was observed in transdifferentiated tissue (Figure 7a, b). The sizes of the digested bands on the gelatin zyogram indicated that the majority of the signal was due to an increase in MMP2 and MMP9 net activity. On the casein gel, the size of the band suggested a possible upregulation of cathepsin activity. The arrays also identified an increased level of Cathepsin Z mRNA expression in transdifferentiated tissue. Cathepsin Z shares close homology to Cathepsin D and L (Sol-Church *et al*., 2000; Turk *et al*., 2000, 2001), which are both able to degrade casein (Dufour and Ribadeau-Dumas, 1988; Larsen *et al*., 1996). Cathepsins Z, D and L are all very close to the size observed in the degradation of the casein substrate, and Northern blot analysis confirmed an increase of Cathepsin Z mRNA in transdifferentiated tissue (Figure 3). Although the Northern blot indicated an upregulation of Cathepsin Z, it was possible that the zymogen activity observed on the casein gel may be due to the presence of Cathepsin D or L. The arrays were able to identifiy Prosaposin as upregulated in the transdifferentiated tissue (Table 5), and this protein has been implicated in sharing a similar expression pattern with Cathepsin D (Campana *et al*., 1999). Northern blot analyses revealed a similar expression pattern for Prosaposin and Cathepsin Z, suggesting that the casein degradation may be due to the expression of more than one enzyme of similar size in this region (Figure 7b).

The zymogram assays using whole tissues indicated that a program had been initiated that leads to an increase in MMP activity. We hypothesize that this MMP activity might have been due to a transient population of cell types involved with the inflammatory response as an effect of β-catenin accumulation. We have not done any RNA localization *in situ* to determine the specific cell type responsible for the observed MMP activation, and this remains a subject for further study.

Discussion

β-Catenin is an integral component of the Wnt signaling cascade and its stabilization specifically in mammary epithelium leads to a loss of differentiation followed by transdifferentiation and the establishment of squamous metaplasias. The molecular state of transdifferentiated tissue at distinct stages of progression was assessed with cDNA microarrays. The use of the Mammochip, which contains mainly unknown genes, facilitated the identification of new genes that are activated during the β-catenin-induced transdifferentiation process. This approach also demonstrated that the activation of β-catenin signaling results in the temporal maintenance of differentiated mammary epithelium after the onset of involution and is accompanied by the maintenance of Stat5a activation as gauged by its nuclear localization. In contrast, no nuclear Stat5a was observed in the tissue that had undergone complete transdifferentiation.

The Mammochip was developed as a tool to discover new genes that are expressed in normal mouse mam-

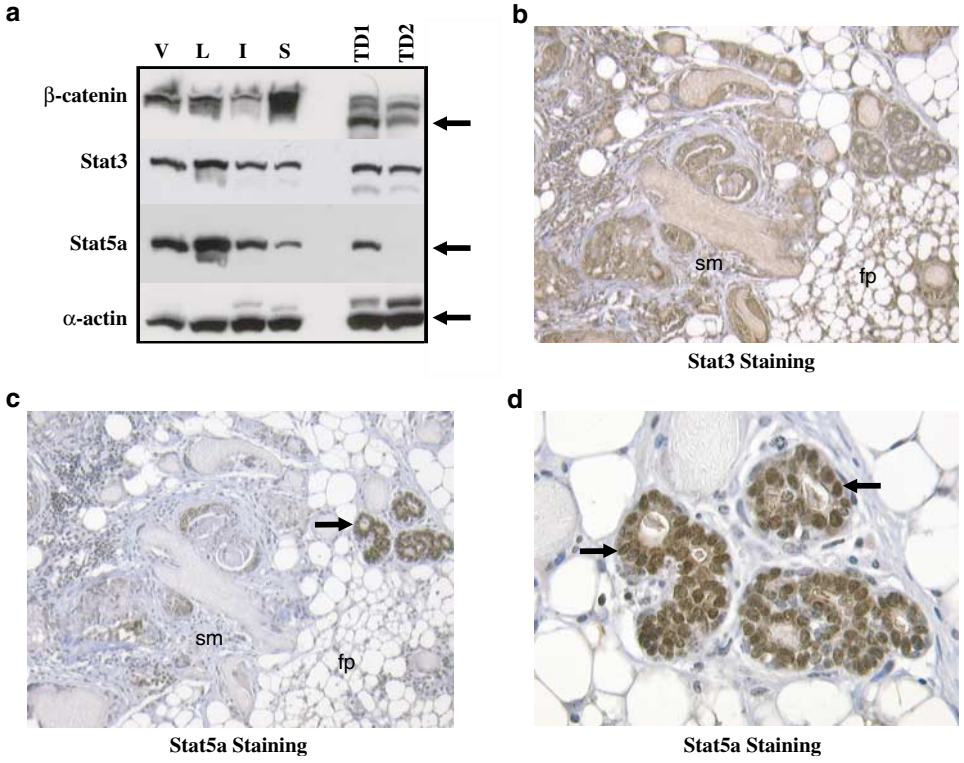

Figure 4 Analysis of Stat3 and Stat5a expression in transdifferentiated tissues from wild-type and Catnb$^{+/\Delta ex3}$:WAP-Cre mice. (**a**) Western blot analysis demonstrating the expression of β-catenin, Stat3, Stat5a and α-actin in mammary tissue from the same tissues used in Figures 2 and 3. The specific stabilization of β-catenin is demonstrated in the transdifferentiated tissues TD1 and TD2 by the appearance of a lower band on the blot indicating a loss of amino acids 5–80. Stat3 expression does not appear to be altered in the tissues analysed and the level of expression in the skin is comparable to both normal mammary tissue and transdifferentiated tissues. Stat5a expression is lost in the highly differentiated tissue TD2, but the expression is maintained at a level comparable to normal mammary gland tissues in the less-transdifferentiated tissue TD1. α-actin expression is similar in all of the tissues used for analysis. Arrows indicate the band corresponding to the respective protein. (**b**) Stat3 immunohistochemistry in mammary tissue from sample TD1. (**c**) Stat5a staining demonstrated the loss of Stat5a in areas that had undergone extensive squamous transdifferentiation, and the retention of both nuclear and cytoplasmic localization in remaining epithelial structures (arrow). (**d**) Higher magnification of alveoli-like structures in mammary tissue from Catnb$^{+/\Delta ex3}$:WAP-Cre mice after 10 weeks of involution. The nuclear localization of Stat5a (arrows) further suggests that milk protein mRNA detected after 10 weeks of involution are the result of β-catenin-mediated maintenance of epithelial differentiation. (fp) indicates an area containing the mammary fat pad and (sm) represents an area of squamous metaplasia

mary tissue and possibly control its development and physiology. The use of this chip not only verified the expression of known milk protein genes in lactating tissue, but also led to the identification of new genes highly expressed during functional differentiation. Importantly, these experiments also revealed that the gene expression profile of involuted tissue does not mimic that observed in mature virgins, and that new genes are activated specifically during the process of involution. This observation confirms on a molecular level that mammary tissue, which has completed one round of functional differentiation followed by complete involution, does not return to its original state. Based on these results we hypothesize that the expression or repression of specific genes may be necessary for the maintenance of involuted epithelium. Alternatively, a change in the cell population, such as a shift in the number of alveolar stem cells, may account for the differences. The identification of genes that distinguish the virgin from the remodeled state provides the basis to further understand these differences and the physiological consequences.

Several important lessons emerged from this study. The analysis of transdifferentiated tissue at different stages of progression led to the establishment of four groups of genes that may indicate common regulatory motifs or similar mechanisms for the relative pathway activation. Notably, on a molecular level, the transdifferentiation process resulted in the activation of epidermal-specific genes. In addition to known genes, many new genes were activated in transdifferentiated tissues. One encodes a 1.8 kbp RNA product that was detected in a restricted set of tissues, including mammary epithelium. The second gene encodes a 1.5 kbp mRNA whose expression was detected in lactating mammary tissue and in transdifferentiated tissue that still contained some epithelium. The only corresponding EST in GenBank is from a mammary library, suggesting an expression limited to mammary tissue. However, increased expression was also detected in prostate tissue that had undergone β-catenin-induced hyperproliferation and transdifferentiation (our results unpublished), suggesting a role in epithelial cell pro-

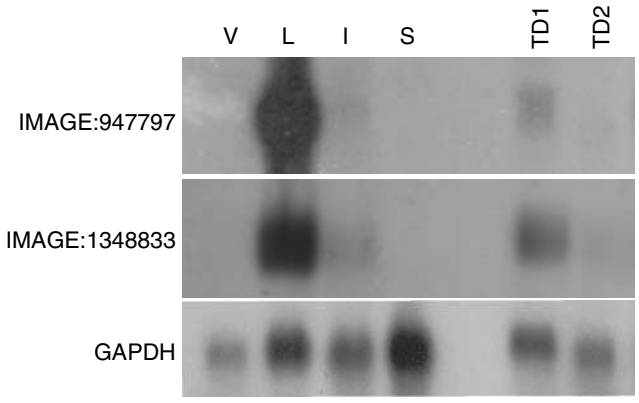

Figure 5 Northern blot analyses of several unknown genes from the Mammochip that were induced in β-catenin-induced transdifferentiated tissue from Catnb$^{+/\Delta ex3}$:WAP-Cre mice as compared to control involuted tissue. The samples used were the same as in Figure 2. High expression of IMAGE clone 947797 was observed in lactating mammary tissue. Expression in sample TD1 was higher than in the involuted control tissue. The expression pattern of IMAGE clone 1348833 was similar to that of clone 947797. GAPDH expression was used as a loading control

liferation or expression by the infiltrating inflammatory cell types. A new gene was identified that encodes a 20 kDa protein product with a distinct subcellular distribution. Based on the nuclear localization of this protein during uninhibited growth in culture, and the preferential expression in highly proliferative tissues as determined by the UNIGENE cluster Hs.208912 distribution, the protein was named PANE1. At this point the functional role of the 20 kDa protein is not known. Although it is conserved between species, no known protein motifs were apparent.

The particular approach used in this study pointed to new genes that may contribute to, or are expressed as a result of β-catenin accumulation. This study also demonstrated an altered expression of basement membrane proteins, or those mediating its maintenance, in transdifferentiated tissue. Specifically, the increase in protease activity may reflect the remodeling process that parallels the transdifferentiation process, and possibly preceding stages of hyperproliferation. A smaller increase of cathepsins has been observed during normal involution using Affimetrix arrays (Chaudhry *et al*., 2002; Master *et al*., 2002). The expression of the extracellular matrix modifiers may be linked to the inflammatory response rather than the direct intracellular signaling because of β-catenin accumulation. Regardless, the tissue compartment as a whole shows increased MMP activity.

Finally, profiling gene expression in normal mammary tissue and upon β-catenin-induced transdifferentiation has emerged as a viable tool to further characterize the state of transdifferentiation. The characterization of newly identified genes and an understanding of their function may continue to shed light on the molecular processes underlying the establishment or maintenance of cell specification and identity in response to the activation of β-catenin.

Material and methods

Mammary tissues

Transgenic mice harboring a stabilized endogenous β-catenin have been described (Miyoshi *et al*., 2002). Briefly, exon 3 of the endogenous *β-catenin* gene (encoding amino acids 5–80) was flanked by loxP sites, and the deletion in differentiating mammary epithelium was accomplished with a Cre transgene under control of the *WAP* gene promoter. Mammary tissues from wild-type mature virgin mice, at day 1 of lactation, and 10 weeks of involution were compared with tissues from transgenic mice at 10 weeks of involution, which exhibited different degrees of metaplasias. The two mutant samples were obtained from transgenic mice that had gone through one pregnancy followed by 10 weeks of involution. We have not observed any adverse effects suggesting toxicity related to Cre expression (Loonstra *et al*., 2001; Pfeifer *et al*., 2001; Silver and Livingston, 2001).

Microarray analysis

The microarray analysis has been performed with both a 2.6 k NCI Oncochip mainly comprised of known genes (Mm OC 6-3; Advanced Technology Center, NCI, NIH), and a mammary-specific chip (Mammochip) comprised of mostly unknown genes. Approximately 6000 unigenes were selected as highly enriched in mammary tissue from two libraries containing a total of 52 000 sequences: Soares NbMMG GenBank ID: 403 9 (virgin) and Soares NMLMG GenBank ID: 636 (lactating). Each sequence was spotted at least twice thus generating a 12.9 k cDNA array: Mm-M-v1p1 (Advanced Technology Center, NCI, NIH), which includes about 18% known genes and 82% unknown genes. The lists are accessible at http://nciarray.nci.nih.gov/gal_files/. RNA was prepared from mammary tissue of control mice virgin, lactation day 1, and 10-week involuted mice. RNA was prepared from β-catenin-stabilized mammary tissues that had undergone 10 weeks of involution and demonstrated differing degrees of transdifferentiation. All RNA was prepared using TRIzol extraction (Life Technologies, Rockville, MD, USA) followed by two ethanol precipitations. For fluorescence labeling, 20 μg of total RNA was reverse transcribed in the presence of 300 U SuperScript II (Life Technologies, Rockville, MD, USA), then labeled with cy3-dUTP and cy5-dUTP (NEN, Boston, MA, USA). Samples were combined, purified and concentrated with YM30 Microcon columns (Millipore, Bedford, MA, USA). Slides were prehybridized for 1 h and hybridized overnight at 42°C, in 25% formamide. Slides were washed in 2× SSC + 0.1% SDS 4′, 1× SSC 4′, 0.2× SSC 4′, 0.05× SSC 1′, and dried by centrifugation. The arrays were scanned on a GenePix 4000A scanner (Axon Instruments, Foster City, CA, USA). The images were analysed by GenePix Pro 3.0 (Axon Instruments, Foster City, CA, USA). Each experiment was repeated with switched labeling. The data were deposited in the NCI-CIT microarray database and normalized data were

Table 6 Analysis of unknown genes upregulated in β-catenin transdifferentiated tissues

IMAGE ID	*Exons*	*Chromosome*	*Open reading frame*	*Protein homology*
(A) Genes upregulated in TD1 and TD2				
820521	No	11	<100 aa	No
850508	Yes	1	Yes	tRNA Synth.
863507	No	14	–	*3′UTR glypican-6 precursor
947725	No	6	Yes	Hypothetical
947821	No	15	<100 aa	No
947854	No	11	–	*3′ UTR unknown gene
1178579	Yes	15	Yes	Hypothetical
1246820	Yes	15	Yes	RNA polymerase III subunit
1313772	Yes	6	Yes	Lectin type C
1348833	Yes	2	<100 aa	No
1363898	No	9	–	*B-gal 3′UTR?
1431010	Yes	8	Yes	Hypothetical
1448863	Yes	5	Yes	Syntaxin 18
1495233	Yes	17	Yes	Hypothetical
1496033	No	11	Yes	Hypothetical
1496241	No	3	Yes	TNIK cytoskel.
1511503	Yes	18	Yes	Hypothetical
(B) Genes upregulated in TD1				
851724	Yes	7	Yes	Nadrin2
862850	No	6	–	*3′ UTR Sideroflexin
947797	No	15	<100 aa	No
1245783	Yes	17	Yes	Zinc Finger Pro.
1384161	No	6	<100 aa	No
14476620	No	11	Yes	Sulfotransferase
1448100	No	10	<100 aa	No
1449282	Yes	14	Yes	No
1493858	No	9	No	No
(C) Genes upregulated in TD2				
820678	Yes	12	Yes	LBP-32
863369	No	17	Yes	Protein Kinase
875125	No	3	<100 aa	No
963317	No	5	Yes	No
1195010	Yes	11	Yes	Vacuole protein
1399551	Yes	9	Yes	MME-12

(D) Image 1178579 represents a highly conserved hypothetical protein

Mus musculus	MSVLRSMDKLPDLNRATVLLVSTEDALLQQLAESMLKDDASELRVHLANSLPLPSNVNR	60
Rattus norvegicus	MSVLRPMDKLPDLNRATILLVSTEDALLQQLAESVLKIDCATELRVHLANSLPLPSNANR	60
Homo sapiens	MSVLRPLDKLPGLNTATILLVGTEDDALLQQLADSMLKEDCASELKVHLAKSLPLPSSVNR	60
	***** **** ** ** *** ********** * ** *** ** **** ****** **	
Mus musculus	PRIDLIVFVINLHSKYSLQKVEEFLQHVDSSFFLGKVCFLVTGAGQESHCSVHQNTVIKL	120
Rattus norvegicus	PRIDLIVFVINLHSKYSLQKVEESLNHVDSSFFLGKVCFLVTGAGRESHCSVHQNTVMKL	120
Homo sapiens	PRIDLIVFVVNLHSKYSLQNTEESLRHVDASFFLGKVCFLATGAGRESHCSIHRHTVVKL	120
	***** **** ** ** *** ********** * ** *** ** **** ****** **	
Mus musculus	AHTYRSPLLLCDLQVESFRAAMARRLVRILQICAGHVPGVSALNLMSLLRSPENPPSKEL	180
Rattus norvegicus	AHTYRSPLLFCDLQVESFRAAMAQRLVRVLQVCAGHVPGISALNLLSLLRSPENPPSKEL	180
Homo sapiens	AHTYQSPLLLCDLEVEGFRATMAQRLVRVLQICAGHVPGVSALNLMSLLRSSEGPSLEDL	180
	**** **** *** ** *** ** *** ** **** ** ******* ***** ***** * * *	

Gene selection for further analysis (**a-d**). IMAGE clones were taken from the array results indicating unknown gene upregulation after microarray analysis. Genes that were upregulated in the less transdifferentiated tissue TD1 (**a**), tissues demonstrating a higher degree of transdifferentiation TD2 (**b**) and those upregulated in both TD1 and TD2 (**c**) were analysed. The analysis is based on bioinformatics using the known regions from each gene and therefore are subject to change after the full-length cDNA sequences are determined for each gene. The column indicating protein homology refers to regions that share at least some homologous region and may represent family members or proteins with functional similarities. The first gene on the list that met our criteria for selection demonstrated a high level of conservation between species (**d**), suggesting a relative biological significance

analysed with mAdb tools (NCI-CIT) using the following parameters: differentially expressed genes were selected with a signal intensity of fivefold higher than the average slide background in at least one channel, and an expression ratio of at least 3 for upregulated genes or 0.3 and lower for downregulated genes. These criteria had to be met in the label and reverse-label experiments for a gene to be included in consideration for analysis of altered expression.

Northern-blot analysis

Total RNA was prepared from fresh whole tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA)

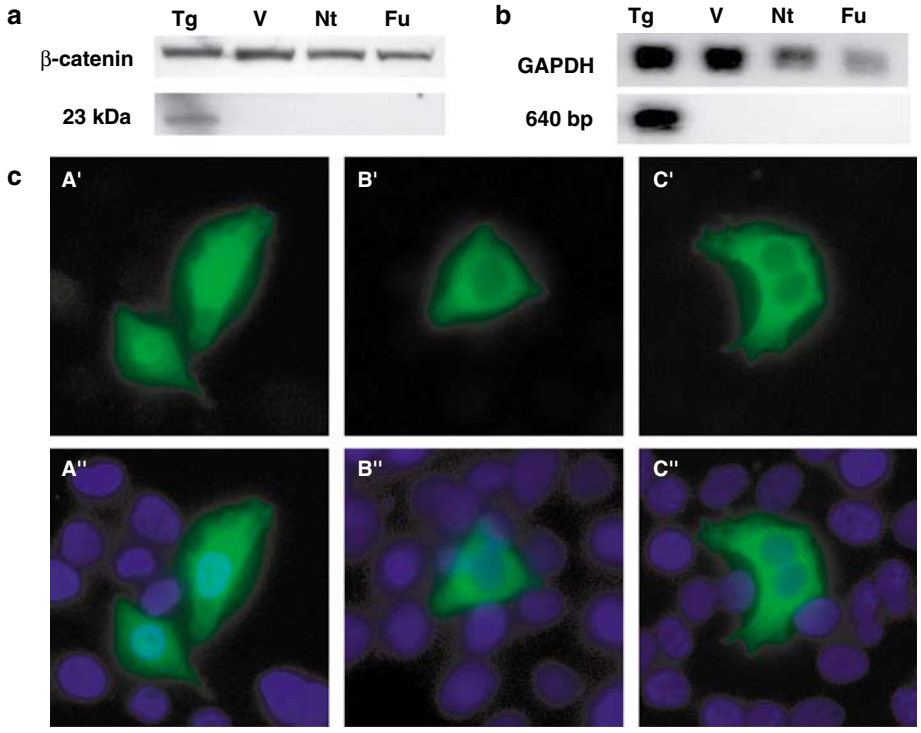

Figure 6 Analysis of the protein encoded by the gene represented by IMAGE clone 1178579 upon transient transfection in HeLa cells. HeLa cells were transfected with an expression vector containing the open reading frame from the gene represented by IMAGE clone 1178579 and a viable protein was generated (**a**). The myc-his fusion protein corresponded to an approximate size of 23 kDa, and supports the predicted size of 20 kDa for the endogenous protein. Endogenous β-catenin was used as a loading control. To further verify that the myc antibody was specific for the fusion protein, a Northern blot was performed to verify the expression of the transfected construct (**b**). GAPDH was used as the loading control. (Tg) vector with insert, (V) empty vector, (Nt) no-transfection control; (Fu) Fugene 6 Transfection Reagent only control. The transfected cells were fixed and analysed by immunofluorescence using the anti-myc antibody (**c**). Clear nuclear localization was observed in the nonconfluent cell populations (**a**′ and **a**″), while exclusion from the nucleus was observed when the culture became confluent (**b**′ and **b**″) or the cells divided (**c**′ and **c**″). It is worth noting that the protein was excluded from the nucleolus when localized within the nucleus (visible in **a**'). DAPI is blue and the myc antibody is visible in green (Alexa Fluor 488 conjugated secondary antibody)

essentially as described by the supplier's protocol. Total RNA (10 μg) was loaded in each well and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Hybrisol 1 solution containing 50% formamide, 10% dextran sulfate, 1% SDS and blocking reagent (Intergen, Purchase, NY, USA) was used for hybridization. Prehyridization was performed at 45°C for 3 h with the Hybrisol 1 solution. Sequences used to amplify the probes were: (IMAGE: 947797) 5′CACCACAGACAGGAGAGCAA3′-F, 5′TGATTTGTTGGGGTTTGTCA3′-R; (IMAGE:1348833, Keratin 5, α-casein, GLTP, and CA III were amplified from image clones using the common T3 and T7 primers) 5′ATTAACCCTCACTAAAGGGA3′-F, 5′TAATACGACTCACTATAGGG; (STE) 5′TCTTTCCTAATC- TGTAGTGCC3′-F, 5′AACCTTTCACACCACCTCA- C3′-R; (FABP4) 5′GCCTTTCTCACCTGGAAGAC3′-F, 5′AAACCTTCTGTGGAAGTCACG3′-R; (Prosaposin) 5′TGTGCCTCCTCAGAAGAATGG3′-F,5′CTAGACCCACAAGTAGGTGAC3′-R; (Cathepsin Z) 5′GCAGACCGAATCAACATCAA3′-F, 5′GCATAG- ATCTCGGCCATCAT3′; (GAPDH) 5′GTGAAGG- TCGGTGTGAACGGATTTGGCCGT3′-F, 5′CCACCACCCTGTTGCTGTAG3′-R. The probe used for the transfection studies was the *Not*1 and *Xba*1 digested fragment from the unligated sequence verified PCR product from the open reading frame of the gene represented by IMAGE clone 1178579. The radio-labeled probes were hybridized at 1.5×10^6 c.p.m./ml for 18 h at 42°C. After hybridization, the blots were washed two times with $2 \times$ SSC + 0.1%SDS, and twice with $0.1 \times$ SSC + 0.1%SDS at 42°C. The blots were exposed at −80°C for 3, 6, 9, 12, 15, and 24 h depending on the gene.

Western blot analysis

Protein extracts for analysis by Western blot were obtained from β-catenin conditionally stabilized females, and lysate was prepared from fresh and frozen

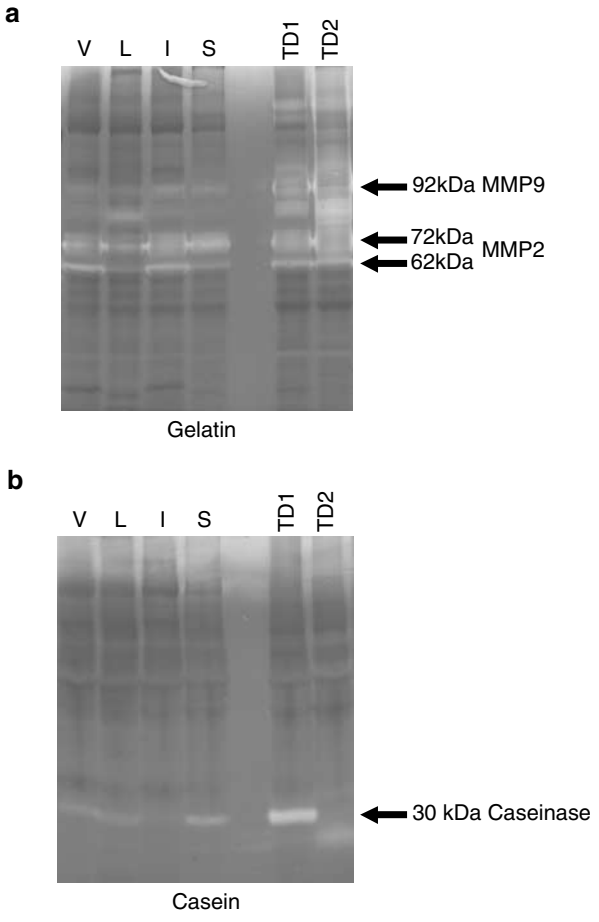

Figure 7 Zymogram analysis of transdifferentiated tissues to investigate protease activation. The samples used were the same as in Figure 2. (**a**) Gelatin embedded SDS–PAGE demonstrating the induction of both MMP9 and MMP2 in samples TD1 and TD2. The upregulation of MMP9 is demonstrated at 92 k in both the transdifferentiated tissues. MMP2 activity observed at 72 and 62 k for the pro and active form, respectively, is higher in the TD2 sample than any of the other tissues analysed. (**b**) Casein embedded SDS–PAGE demonstrating degradation of the casein substrate at 30 kDa, suggesting an increase in protease activity that may include several of the Cathepsin family members. In contrast to the gelatin degrading proteases that are able to degrade type IV collagen as found in the basement membrane of wild-type mammary epithelium, the observed 30 kDa proteinase activity is expressed at a much higher level in the less transdifferentiated tissue TD1. Activity of the 30 k casein degrading protease was also observed in the skin

tissues. Tissue was homogenized protein lysis buffer containing the proteinase inhibitors aprotinin, leupeptin and PMSF at 50 μg/ml. The activated phosphotyrosyl proteinase inhibitor sodium orthovanadate was prepared, and added to a final concentration of 4 mM. Total protein (40 μg) in 1 × Tris-glycine SDS loading buffer (Invitrogen, Carlsbad, CA, USA) with BME was loaded on 8% Tris-glycine SDS gels (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. After the gel electrophoresis, proteins were transferred to PVDF membranes. Antibodies were diluted in 2.5% BSA blocking buffer at 1 : 500 for β-catenin (C-19220; Transduction Laboratories, San Diego, CA, USA) and α-actin (05-384; Upstate Biotechnology, Lake Placid, NY, USA); 1 : 1000 for Stat3 (SC-482; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and 1 : 5000 for Stat5a (Liu *et al*., 1996). The primary antibodies were allowed to incubate at room temperature for 1 h, then the membranes were washed for 5 min 3 × using 1 × TBST. Secondary antibody was diluted at 1 : 5000 and incubated for 45 min, and after incubation the membranes were washed for 10 min 5 × using 1 × TBST. HRP was detected using the Super Signal Western Detection Kit (Pierce Chemical, Rockford, IL, USA) as described by the manufacturer's protocol. In addition to α-actin as a control, loading was determined by Coomassie staining (Pierce Chemical, Rockford, IL, USA) of parallel gels.

Immunostaining

Tissues were fixed in 10% NBF at 4°C overnight. After fixing, the tissues were transferred to 70% alcohol and sectioned to a depth of 5 m. After deparaffinization and rehydration, the endogenous peroxidase activity was blocked using 10% methanol + 0.3% H_2O_2 for 20 min followed by two washes in 1 × PBS. Antigen unmasking solution (Vector Laboratories Incorporated, Burlingame, CA, USA) was used for antigen retrieval. The unmasking solution was diluted 1 : 100 in distilled water and the sections were microwaved in 600 ml of this solution for 20 min. The sections were blocked in 1 × PBS + 3% normal goat serum for 30 min at room temperature. The Stat5a antibody that has been previously described (Liu *et al*., 1996) and a Stat3 (SC-482; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody were diluted in blocking solution at 1 : 600 and 1 : 400 respectively then allowed to incubate at 4°C overnight. The slides were washed twice in 1 × PBS, and the secondary antibody (Vector Laboratories Incorporated, Burlingame, CA, USA) was applied to the sections for 1 h at room temperature. After incubation with the secondary antibody, ABC reagent was added for 30 minutes at room temperature. (ABC kit; Vector Laboratories Incorporated, Burlingame, CA, USA). To detect staining, DAB peroxidase substrate (Vector Laboratories Incorporated, Burlingame, CA, USA) solution was added as described in the manufacturer's protocol (excluding addition of the nickel solution). The sections were allowed to incubate until staining was observed under the microscope. The sections were counterstained with hematoxylin using a standard protocol.

Cloning and transient transfections

IMAGE clone 1178579 was localized to a genomic region that corresponded to the RIKEN cDNA 2610019I03 clone (GenBank AK011464). All of the PCR reactions were performed using High Fidelity Taq (Invitrogen, Carlsbad, CA, USA). The primers used to amplify 41–690 bp (GenBank AY243536, AY243537)

from the RIKEN sequence were: 5′CTGGAGCCGAGTGGCGCTGC-F and 5′CCTGTCAGGCCCTGACTGGTC-R. The PCR product was amplified with a 55°C annealing temperature for 35 cycles using lactating mammary gland and TD1 RT reaction templates. The lactating mammary gland template was used to ensure that this gene could be amplified using a common tissue source (rather than only the TD1 sample). The 5′ *Not*1 and 3′ *Xba*1 overhangs were produced using a nested PCR from the sequence verified product in the previous reaction. The primers used for the sticky overhangs of the open reading frame were: 5′ATAAGAAGGCGGCCGCATGTCGGTGCTGAGTGCTGAGGTCGATGG-F and 5′CTAGTCTAGACAGCTCCTTGGATGGGGGGTTC-R. The PCR products were cut with *Not*1 and *Xba*1 (New England Biolabs, Beverly, MA, USA) for 2 h at 37°C and then gel purified. The pcDNA Version A vector was digested with *Not*1 and *Xba*1 for 2 h and then placed at 65°C for 15 min to stop the reaction. The vector was dephosphorylated using 2 μl of calf intestinal alkaline phosphatase (Boehringer Mannheim, Germany) and the buffer provided within the kit. The vector was heated to 65°C for 15 min to stop the dephosphorylation reaction and the vector was gel purified. The ligation was performed with T4 DNA ligase (1 μl from a 400 000 U/ml stock and buffered with the solution recommended by the manufacturer; New England Biolabs, Beverly, MA, USA) for 20 min at room temperature using 50 ng of vector and 21 ng of each purified sticky PCR product (1 : 3 vector to insert molar ratio) as described by the manufacturer. Top10 Oneshot chemically competent *Escherichia coli* cells (Invitrogen, Carlsbad, CA, USA) were transformed with 5 μl of the ligation product as outlined in the protocol provided by the manufacturer. Positive colonies were screened by PCR using the following primers: 5′TAAT ACGACTCACTATAGGG-F (T7 from the vector) and 5′CTGCAGTGGCTTTCCTGTC-R (near the midpoint inside the open reading frame). All of the clones were sequence verified using the T7 and BGH sites from the vector. The transient transfections were performed in HeLa cells (ATCC, Manassas, VA, USA) using the Fugene 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA) as described by the manufacturer. All experiments were performed in triplicate and three individual clones containing the insert were used to ensure reproducibility of the results. Prior to each set of transfection experiments, the entire stock of trypsinized HeLa cells were pooled then an equal volume of the trypsinized cell suspension was delivered to each culture dish. Each transfection was performed identically in three dishes. At 48 h, one dish was collected for protein, one for RNA and the third dish was then trypsinized and the cells were passed to individual chambered slide compartments (Nalge Nunc International Corporation, Naperville, IL, USA). In each chamber, the cells were added such that the next morning the chambers would display varying degrees of confluence from around 50, 75, 100% and overgrown. The medium was removed and the cells were washed with 1xPBS. Neutral buffered formalin (NBF) (10%) was used to fix the cells for 30 min at room temperature. The cells were washed three times with 1 × PBS and then the primary mouse Anti-myc (Invitrogen, Carlsbad, CA, USA) antibody was added (1 : 1000 suspended in 1 × PBS with 3% goat serum) and allowed to incubate for 1 h at room temperature. The primary antibody was removed and the slides were washed three times with 1 × PBS. The Alexa Fluor 488 conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR, USA) was used at 1 : 400 (suspended in 1 × PBS with 3% goat serum) for 45 min at room temperature. The slides were then washed two times with 1 × PBS and mounted with the DAPI Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA).

Zymogram analysis

The zymography was performed on 10% gelatin and 12% casein embedded Tris-glycine SDS gels. The zymogram gels have been performed essentially as described in the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA), and the lysate preparation was the same as previously described in the Western analysis section. Protein (40 μg) from the whole gland lysate was loaded in each lane. The gel was allowed to electrophorese, then Zymogram Renaturing Buffer (Invitrogen, Carlsbad, CA, USA) was added for 30 min at room temperature. After 30 min, the Zymogram Developing Buffer (Invitrogen, Carlsbad, CA, USA) was added, and the gels were allowed to incubate at room temperature for 30 min. The Zymogram Developing Buffer was discarded and replenished with fresh buffer, then allowed to incubate at 37°C for 24 h. The gels were Coomassie stained, then dried. The protease activity was visualized by the appearance of clear bands that indicated the digestion of gelatin and casein substrates, respectively, embedded within the gels. Relative molecular sizes for the digested bands were determined using molecular weight markers and interpretation was performed using Alpha Imager software.

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